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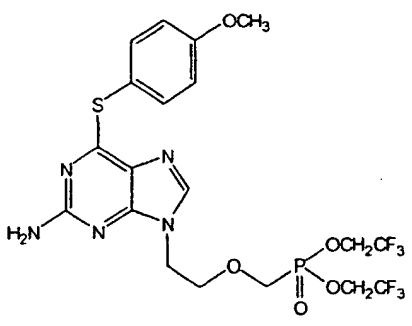
(54) Title: TREATMENT OF PRE-CORE HEPATITIS B VIRUS MUTANT INFECTIONS



(I)

(57) Abstract: Provided are medicaments and methods for preventing or treating pre-core mutant hepatitis B virus disease, and lamivudine-resistant, pre-core mutant hepatitis B virus disease, in human patients comprising administering to a patient in need thereof a therapeutically effective amount of 2-amino-9-[2-[bis(2,2,2-trifluoroethoxy)phosphonylmethoxy]ethyl]-6-(4-methoxy-phenylthio) purine .1

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TREATMENT OF PRE-CORE HEPATITIS B VIRUS MUTANT INFECTIONS

This application claims the benefit of priority of U.S. Provisional Application Serial No. 60/365,425, filed March 18, 2002; U.S. Provisional Application Serial No. 60/372,719, filed April 12, 2002; U.S. Provisional Application Serial No. 60/383,852, filed May 28, 2002; and U.S. Provisional Application Serial No. 60/411,559, filed September 17, 2002, the contents of each of which are herein incorporated by reference in their entirety.

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to methods for preventing or treating pre-core mutant hepatitis B virus (HBV) infections in human patients, including pre-core mutant hepatitis B virus infections that also exhibit resistance to lamivudine ((-)- β –L-2',3'-dideoxy-3'- thiacytidine, also referred to as 3TC), a conventional hepatitis B virus antiviral agent.

Description of Related Art

Hepatitis B virus infections are considered to be a medically crucial problem because of the large number of afflicted patients world-wide. For the purpose of treating this disease, various agents having antiviral activity have been developed. Among these, lamivudine was developed as the first antiviral agent that inhibits the replication of HBV. Lamivudine has recently been approved for treating HBV infection in several countries, and there are many reports regarding the clinical use of this drug. A serious problem associated with the treatment of HBV infections with lamivudine discussed in these reports is the emergence of lamivudine-resistant viruses

that increase after the long-term administration of this drug.

Lamivudine-Resistant HBV Mutants

Lamivudine was the first antiviral agent effective in inhibiting the replication of both HIV and HBV. It has already been approved as an agent for HIV and HBV infection in many countries.

As noted above, the appearance of lamivudine-resistant HBV increases after the long term administration of this drug. As in the case of HIV, lamivudine-resistant HBV exhibit an alteration of methionine to valine or isoleucine in the amino acid sequence motif "tyrosine-methionine-aspartic acid-aspartic acid" (YMDD) located in the active site of both the HIV reverse transcriptase and the HBV DNA polymerase, which also exhibits reverse transcriptase activity.

In the case of HIV, X-ray crystallographic analysis of a YMDD mutant reverse transcriptase suggests that the substitution of methionine by valine or isoleucine interferes with the binding of lamivudine triphosphate to the active center and the possibility of cross-resistance to other nucleoside derivatives having β- or L-ring configurations (Sarafianos et al., *Proc. Natl. Acad. Sci. USA* 96: 10027-10032 (1999)).

No X-ray crystallographic structure of the HBV DNA polymerase is presently available. However, because of sequence similarities between the HBV DNA polymerase and the HIV reverse transcriptase, knowledge regarding the genotypic resistance of HIV reverse transcriptase to lamivudine can be applied to the HBV DNA polymerase. For example, alterations analogous to those seen in the YMDD motif of HIV reverse transcriptase conferring resistance to lamivudine have also been noted in the YMDD motif of HBV DNA polymerase (A. Bartholomeuz and S. Locarnini, Mutations in the Hepatitis B Virus Polymerase Gene that are Associated with Resistance to Famciclovir and Lamivudine, *International Antiviral News*, Vol. 5(8), 123-124 (1997)). Furthermore, in regions other than the YMDD motif, analogous mutations conferring resistance to famciclovir as well as lamivudine have been noted for both HIV and HBV. *Id.*

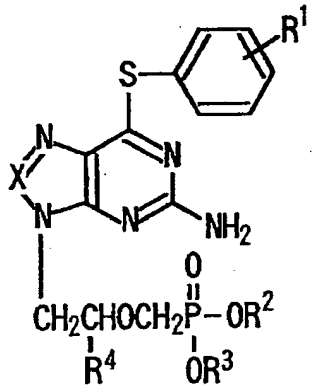
Pre-Core HBV Mutants

The hepatitis B virus virion consists of an outer lipoprotein layer and an icosahedral core containing a shell of core protein, polymerase, and the HBV DNA genome. The genomic DNA is partially double stranded and circular. There are four major open reading frames (genes): S (surface), C (core), P (polymerase), and X (transcriptional transactivating). The S gene consists of three regions: pre-S1, pre-S2, and the surface proteins (HBsAg). Very rarely, a mutation may occur in the S gene and may abort the HBsAg, with the result that a person may be HBsAg negative but still be infected with virus as evidenced by the presence of HBV DNA. The C gene is divided into two regions: the pre-core and the core, and codes for two different proteins, the e antigen (HBeAg) and the Core antigen (HBcAg), respectively. A common HBV mutant is the pre-core mutant, in which the processing of the HBeAg does not occur, resulting in the absence of the e antigen. An example of a pre-core mutation is one in which there is a substitution of glycine for alanine at amino acid position 1896 of the pre-core protein. Patients chronically infected with the HBV pre-core mutant will be HBsAg positive, HBV DNA positive, and HBeAg negative. A third mutant with another alteration in the core has been described and is referred to as HBV2 (Coursaget et al., Hepatitis B Surface Antigen Reactivity in Man Due to a New Variant of Hepatitis B Virus, *Vaccine,* Vol. 8, Supp S15-7 (1990)). Patients infected with these mutant viruses are HBsAg positive, but lack HBeAg and do not have antibodies to HBeAg.

Because of the existence of pre-core mutant HBV, and pre-core mutant HBV that are also lamivudine-resistant, the effectiveness of lamivudine as an antiviral agent is limited, and there is a need for additional antivirals to treat patients chronically infected with such mutant viruses.

SUMMARY OF THE INVENTION

The present invention provides methods for preventing or treating pre-core mutant hepatitis B virus disease, which comprises administering to a patient in need thereof an antiviral effective amount of a phosphonate nucleotide compound represented by the following formula (I):



(I)

wherein:

R^1 is a hydrogen atom, a C_1-C_6 alkoxy group, a C_1-C_4 alkoxy group substituted by one or more halogen atoms, a halogen atom, an amino group, a hydroxyl group, or a nitro group;

each of R^2 and R^3 is independently a hydrogen atom, a C_1-C_{22} alkyl group, an acyloxymethyl group, an acylthioethyl group, or an ethyl group substituted by one or more halogen atoms;

R^4 is a hydrogen atom, a C_1-C_4 alkyl group, a C_1-C_4 hydroxyalkyl group, or a C_1-C_4 alkyl group substituted by one or more halogen atoms; and

X is CH or a nitrogen atom,

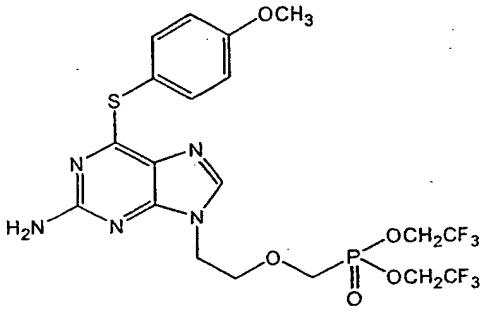
or a pharmaceutically acceptable salt, hydrate, or solvate thereof.

The present invention also provides methods for preventing or treating lamivudine-resistant, pre-core mutant hepatitis B virus disease, which comprises administering to a patient in need thereof an antiviral effective amount of a phosphonate nucleotide compound represented by formula (I), or a pharmaceutically acceptable salt, hydrate, or solvate thereof.

The present invention also provides the use of a compound of formula (I) or a pharmaceutically acceptable salt, hydrate, or solvate thereof, for the manufacture of a medicament for the treatment of pre-core mutant hepatitis B virus disease.

The present invention further provides the use of a compound of formula (I) or a pharmaceutically acceptable salt, hydrate, or solvate thereof, for the manufacture of a medicament for the treatment of lamivudine-resistant, pre-core mutant hepatitis B virus disease.

A preferred compound for use in all the medicaments and methods disclosed herein is:



2-amino-9-[2-[bis(2,2,2-trifluoroethoxy)phosphonylmethoxy]ethyl]-6-(4-methoxy-phenylthio) purine

For convenience, this compound will subsequently be referred to herein as Compound LY.

Further scope of the applicability of the present invention will become apparent from the detailed description provided below. However, it should be understood that the detailed and specific examples, while indicating preferred embodiments of the present invention, are given by way of illustration only since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

DETAILED DESCRIPTION OF THE INVENTION

The following detailed description of the invention is provided to aid those skilled in the art practicing the present invention. Even so, the following detailed description should not be construed to unduly limit the present invention as modifications and variations in the embodiments discussed herein can be made by those of ordinary skill in the art without departing from the spirit or scope of the present inventive discovery.

The contents of each of the references cited herein are herein incorporated by reference in their entirety.

The present inventors have discovered that a 2-amino-6-arylthiopurine-phosphonate, i.e., Compound LY, exhibits a high level of inhibitory activity against the proliferation of pre-core mutant HBV and pre-core mutant HBV that are simultaneously resistant to lamivudine.

Antiviral nucleoside/nucleotide analogs of the naturally occurring nucleosides or nucleotides of adenine, guanine, cytosine, thymine, or uracil with activity against HBV would be expected to exhibit antiviral activity against pre-core (HBeAg negative) mutant HBV that contain a wild-type DNA polymerase as the antiviral mechanism of action of most nucleoside/nucleotide analogs is mediated through the viral DNA polymerase. Such activity results in the disruption of viral nucleic acid replication (J.S. Oxford and B. Oberg, *Conquest of Viral Diseases, A Topical Review of Drugs and Vaccines*, 93-126 (1985)) and therefore of viral replication. As Compound LY disclosed herein is active against lamivudine-resistant, HBeAg positive HBV (J. Colacino, The Purine Nucleotide Analogue LY582563 (MCC-478) Inhibits Replication of Wild Type and Drug Resistant Hepatitis B Virus, *European Association for the Study of the Liver*, Madrid, April 2002; S. Kioko Ono-Nito et al., Novel Nucleoside Analogue MCC-478 (LY582563) Is Effective against Wild-Type or Lamivudine-Resistant Hepatitis B Virus, *Antimicrobial Agents and Chemotherapy*, Vol. 46(8), 2602-2605 (2002)), one might also expect it to be active against lamivudine-resistant, pre-core mutant (HBeAg negative) HBV. However, the effectiveness of Compound LY in

preventing or treating infections caused by pre-core and pre-core, lamivudine-resistant HBV is unexpected as there is evidence indicating that the antiviral mechanism of action of this compound appears to be unlike that of conventional HBV nucleoside/nucleotide analog antivirals.

First, unlike the purine nucleotide analog adefovir dipivoxil, Compound LY exhibits no activity against HIV (Kamiya et al., Antiviral Activities of MCC-478, a Novel and Specific Inhibitor of Hepatitis B Virus, *Antimicrob. Agents Chemother.* 46(9):2872-7 (2002)).

Secondly, as shown in Example 1 below, a diphosphate derivative of an intracellular metabolite of Compound LY does not inhibit the reverse transcriptase activity of endogenous HBV DNA polymerase, while the diphosphate derivative of adefovir and the triphosphate derivative of lamivudine are inhibitory.

The foregoing observations suggest that Compound LY appears to have a mechanism of action against HBV that is different from that of either adefovir or lamivudine, i.e., it does not act like a conventional HBV antiviral nucleoside/nucleotide analog. At present, the precise mechanism of action of Compound LY is unknown. Compound LY may exert its antiviral activity against HBV through viral functions other than HBV DNA polymerase, perhaps affecting encapsidation and packaging of the viral nucleic acid, or digestion of the viral RNA via viral RNaseH. If, for example, Compound LY affects encapsidation and packaging of the viral genome and the function of the core protein, one could not have predicted that this compound would have activity against pre-core mutant (HBeAg negative) HBV, or pre-core mutant HBV that is also lamivudine-resistant.

Processes to prepare Compound LY are disclosed in U.S. Patent No. 5,840,716 and European Patent 0785208. This compound may exist as a salt, and any use of the salts formed by this compound fall within the scope of the present invention. Examples of such salts include pharmaceutically acceptable salts. Where an acidic group exists, the acidic group is able to form metal salts such as a lithium salt, a sodium salt, a potassium salt, a magnesium salt, a calcium salt, an ammonium salt such as an

ammonium salt, a methyl ammonium salt, a dimethyl ammonium salt, a trimethyl ammonium salt, and a dicyclohexyl ammonium salt. Where an amino group exists, the amino group is able to form mineral acid salts such as hydrochloride, hydrobromide, sulfate, nitrate, phosphate, and metaphosphate, and organic acid salts such as methanesulfonate, benzenesulfonate, para-toluenesulfonate, acetate, propionate, tartrate, fumarate, maleate, malate, oxalate, succinate, citrate, benzoate, mandelate, cinnamate, lactate, besylate, valerate, stearate, oleate, lactobionate, ethylsuccinate, semisuccinate, butyrate, palmitate, carbamate, gluconate, laurate, salicylate, tannate, and butylsulfonate.

Phosphonate nucleotide Compound LY and salts thereof may exist in the form of a hydrate or a solvate. Any hydrate or solvate formed by Compound LY and a salt thereof falls within the scope of the present invention. Examples of a solvent capable of forming the solvate include methanol, ethanol, isopropanol, acetone, ethyl acetate, methylene chloride, diisopropyl ether, and the like.

The compound of the present invention is useful as an active ingredient for a pharmaceutical, and particularly for use in preventing or treating infections caused by pre-core-mutation bearing hepatitis B virus, or pre-core mutation/lamivudine-resistant hepatitis B virus. "Effective amount" or "antiviral effective amount" of Compound LY refers to an amount of this compound, or a composition of the present invention, effective to produce the desired or indicated therapeutic effect, e.g., amelioration, retardation, inhibition, reversal, etc. of HBV infection.

When the compound of the present invention is used as a pharmaceutical agent, it can be administered alone, and it is preferred that, using a pharmaceutically acceptable additive, a pharmaceutical composition comprising such compound as an active ingredient is prepared and administered. The composition of the pharmaceutical composition is determined by the solubility of the compound, its chemical properties, administration route, dosage regimen, and the like. For example, Compound LY of the present invention can be orally administered, taking the dosage form of a granule, a parvule, a powder, a tablet, a hard syrup, a soft capsule, a troche, a syrup, an emulsion, a

soft gelatine capsule, a gel, a paste, a suspension, a liposome, and the like, or the compound can be administered intravenously, intramuscularly, or subcutaneously in the form of an injection. Also, powders for injection can be prepared from Compound LY of the present invention so that a parenteral solution can be prepared before using.

As a pharmaceutically acceptable additive, an organic or inorganic, solid or liquid carrier, which is suitable for an oral, enteral, parenteral or local administration, can be used. Examples of a solid carrier used for the preparation of a solid formulation include lactose, sucrose, starch, talc, cellulose, dextrin, kaoline, calcium carbonate, agar, pectin, stearic acid, magnesium stearate, lecithin, sodium chloride, and the like. Examples of a liquid carrier used for the preparation of a liquid formulation for oral administration include glycerine, peanut oil, polyvinylpyrrolidone, olive oil, ethanol, benzyl alcohol, propylene glycol, physiological saline, water, and the like. The above pharmaceutical composition can also comprise, in addition to the above carriers, an auxiliary agent such as a wetting agent, a suspension aid, a sweetener, a flavor, a coloring agent, and a preservative. Further, for use as a liquid agent, it may be contained in a capsule of a substance which can be absorbed such as gelatin. Examples of a solvent or a suspending agent which is used for the preparation of a formulation for parenteral administration such as an injection, include water, propylene glycol, polyethylene glycol, benzyl alcohol, ethyl oleate, lecithin, and the like.

Compound LY exhibits high oral absorbancy. Therefore, oral administration is a preferred administration route for the pharmaceutical compositions of the present invention. The preparation of each of the above agents can be carried out according to standard techniques. Where the agent of the present invention is used for oral administration, the clinical dose can be in the range of from about 0.1 mg to about 500 mg of the compound per kg adult per day, and preferably from about 1 mg to about 50 mg of the compound per kg adult per day. This dose may be modified as appropriate, depending on the patient's age, disease condition, symptoms, the presence or absence of concurrent administration, and the like. The above dose may be administered once per day, or divided over two to several administrations per day at

regular intervals, or may also be applied intermittently every several days. Where the compound of the present invention is used in an injection, the dose can be in the range of from about 0.01 mg to about 50 mg of the compound per kg adult per day, preferably from about 0.1 mg to about 5 mg per kg per day.

Prophylactic modalities for high risk individuals are also encompassed by the present invention. As used herein, the term "high risk individual" is meant to refer to an individual for whom it has been determined, via, e.g., living or working environment or conditions, intravenous drug use, past history of blood transfusion, etc., that there is a significantly higher than normal probability of being susceptible to, or contracting, a hepatitis B virus infection, or the onset or recurrence of an HBV-related disease or disorder. As part of a treatment regimen for a high risk individual, the individual can be prophylactically treated to prevent infection or the onset or recurrence of the disease, disorder, symptom, sign, or condition associated with HBV infection. The term "prophylactically effective amount" is meant to refer to an amount of a pharmaceutical composition of the present invention that produces an effect observed as the prevention of HBV infection, or the onset or recurrence of a disease, disorder, symptom, sign, or condition associated therewith. Prophylactically effective amounts of a pharmaceutical composition are typically determined by the effect they have compared to the effect observed when a second pharmaceutical composition lacking the active agent is administered to a similarly situated individual.

The following examples are provided to illustrate various aspects of the present invention, and should not be construed to be limiting thereof in any way.

Example 1

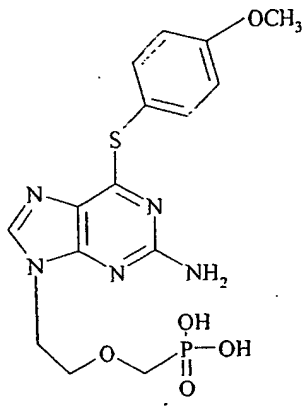
Effects of

a Phosphorylated Compound LY Metabolite, Adefovir Diphosphate, and Lamivudine Triphosphate

on HBV DNA Polymerase Reverse Transcriptase Activity

An intracellular monophosphate metabolite of Compound LY is GCD-189p,

having the structure:



This experiment demonstrates that a phosphate derivative of this monophosphate metabolite, designated GCD-189pp (a diphosphate), does not inhibit the reverse transcriptase activity of endogenous HBV DNA polymerase, while the phosphorylated derivative of adefovir diphosphate, corresponding to the triphosphate of adefovir, and lamivudine triphosphate, are inhibitory. This is evidence that the mechanism of action of an intracellular metabolite of Compound LY in inhibiting HBV replication differs from that of both adefovir diphosphate and lamivudine.

Preparation of Stock Solutions

The phosphorylated metabolite of Compound LY (GCD-189pp) is synthesized by Mitsubishi Pharma Corporation, Tokyo, Japan, and supplied by Eli Lilly and Company, Indianapolis, IN, USA). Adefovir diphosphate (PMEApp) and lamivudine triphosphate (LMV-TP) are obtained from Moravek Biochemicals, Brea, CA. The compounds are prepared as 5mM stock solutions in 100% dimethylsulfoxide (DMSO). Stock solutions of these nucleotide analogues are stored frozen in small aliquots at –20°C, and are diluted before use with nuclease-free water. Trisodium phosphonoformate (foscarnet; Sigma Chemical Company, St. Louis, MO) and [α-^{32}P]-labelled deoxynucleotide triphosphates (dNTPs; supplied as an equimolar mixuture of dATP, dCTP, dGTP and dTTP; Sp. Act >3000 Ci/mMol; Cat. #23030X) are obtained from ICN Biomedicals Inc., Irvine, CA. All other reagents and chemicals

are analytical grade or the highest grade commercially obtainable.

HBV DNA Polymerase

Isolated, partially purified intracellular HBV core particles are used as the source of endogenous HBV DNA polymerase. These particles are generated in cell culture in HepG2 cells by transfecting the cells with a recombinant plasmid containing a full-length copy of the HBV genome under control of the CMV promoter (D.A. Fallows and S.P. Goff, Mutations in the Epsilon Sequences of Human Hepatitis B Virus Affect Both RNA Encapsidation and Reverse Transcription, *J. Virol.* 69:3067-3073 (1995)). Detailed methods for their production, isolation, and purification have been published (Shaw et al., Inhibition of Hepatitis B Virus DNA Polymerase by Enantiomers of Penciclovir Triphosphate and Metabolic Basis for Selective Inhibition of HBV Replication by Penciclovir, *Hepatology* 24:996-1002 (1996)). The enzyme stock is stored frozen in small aliquots at –70°C. In preliminary assays in the absence of inhibitors, the incorporation of [^{32}P]-dNTPs is linear for at least four hours under the conditions of the assay. Several enzymatic activities are associated with the HBV DNA polymerase enzyme. The main polymerase activity associated with intracellular core particles is reverse transcriptase (RT). The same enzyme preparation is used for all assays.

Assay Procedure

The polymerase assay mixture contains 45 µl of enzyme preparation in polymerase buffer (50mM Tris-HCl, pH 7.4, 75mM NH_4Cl, 1mM EDTA, 20mM $MgCl_2$, 0.1 mM β-mercaptoethanol, 0.2% (v/v) NP-40) and 5 µl nuclease-free water (with or without test dNTP analogues or foscarnet). Polymerase reactions are initiated by the addition of 1 µl [α-^{32}P]-dNTP mixture. After four hours of incubation at 37°C, the reactions are stopped by addition of 5 µl 10% (w/v) sodium dodecyl sulfate (SDS) and 2 µl Proteinase K solution (final concentration: 2.5 mg/ml). HBV DNA is then extracted with phenol:chloroform, precipitated with isopropanol, redissolved, and

electrophoresed through 1% agarose gels. The gels are dried and autoradiographed at –70°C with the aid of intensifying screens. Three separate sets of assays are performed. LMV-TP, PMEApp, and GCD-189pp are tested in parallel in each set. The final concentration of each [α-^{32}P]-dNTP in the reaction mixture is approximately 0.0017 µM. Each antiviral dNTP analogue is tested at five different (final) concentrations: 0.005, 0.05, 0.5, 5.0, and 50.0 µM. Foscarnet, a non-nucleotide RT inhibitor (50 µM final concentration) is included in each set of assays as positive control.

Data Analysis

Appropriately exposed autoradiographs are scanned using a UMAX Astra2400s scanner in transmittance mode. Images are analyzed with the aid of SigmaGel, a PC software program from Jandel Scientific, San Rafael, CA. After subtraction of background, the image intensity in each gel lane corresponding to the single-stranded HBV DNA is expressed as a percentage of the image intensity in the corresponding drug-free control lane. TableCurve2D, a statistical/curve-fitting PC program (also from Jandel Scientific, San Rafael, CA) is used to analyze the resulting data and, where possible, to plot individual dose-response curves by unweighted non-linear regression analysis. Where possible, the concentrations that cause a 50% reduction in the amount of [^{32}-dNTP] incorporated into HBV DNA during the four hour assay period (IC_{50}s) are estimated from individual dose-response plots. The results of these experiments are presented in Table 1.

Table 1: Inhibition of Endogenous HBV DNA Polymerase Activity by Different Antiviral dNTP Analogs

	$[^{32}P]$-dNTP Incorporation in the Presence of Analog		
Concentration (μM)	**LMV-TP**	**PMEApp**	**GCD-189pp**
		Assay 1	
Drug-free control	100	100	100
+ Control (foscarnet 50)	0	0	0
0.005	80	120	107
0.05	55	95	110
0.5	2	3	111
5.0	1	1	111
50.0	0	0	70
IC_{50} Estimate (μM)	0.06	0.16	>100
		Assay 2	
Drug-free control	100	100	100
+ Control (foscarnet 50)	37	32	22
0.005	165	100	106
0.05	118	116	89
0.5	30	30	102
5.0	8	18	93
50.0	5	5	55
IC_{50} Estimate	0.26	0.29	58
		Assay 3	
Drug-free control	100	100	100
+ Control (foscarnet 50)	26	25	0
0.005	240	103	138
0.05	130	106	101
0.5	28	24	116
5.0	7	19	131
50.0	5	9	119
IC_{50} Estimate	0.22	0.29	NDR

Data are standardized to drug-free control value of 100%. Unweighted data are fitted by non-linear least squares regression analysis to find the best fit kinetic decay equation by using TableCurve2D. Raw data and IC_{50} estimates (where possible) are

tabulated above.

As shown in Table 1, in each set of assays, 50 μM foscarnet reduces HBV polymerase activity to near-background levels, and both LMV-TP and PMEApp cause dose-related reductions in dNTP incorporation, with IC_{50}s of approximately 0.18 ± 0.11 and 0.25 ± 0.08 μM (mean ± standard deviation), respectively. In contrast, no convincing dose-response relationship is observed in any of the experiments for the phosphorylated Compound LY metabolite GCD-189pp over the concentration range tested (0.005 – 50.0 μM).

Preliminary experiments indicate that the monophosphorylated metabolite of Compound LY, i.e., GCD-189p, does not appear to be active at 50 μM, the only concentration tested in the *in vitro* assays for HBV RT activity described above (results not shown).

Conclusions

These results demonstrate that the phosphorylated metabolite of Compound LY, i.e., GCD-189pp, does not inhibit the reverse transcriptase activity associated with HBV DNA polymerase under experimental conditions in which PMEApp and LMV-TP inhibit this activity. On the basis of previous experience with other antiviral dNTP analogs, it is unlikely that GCD-189pp will prove to be an inhibitor of HBV DNA-dependent DNA polymerase activity, since most of the inhibitors identified to date inhibit both enzymatic activities with approximately equal efficiency. These results suggest that the mechanism of action of this intracellular metabolite of Compound LY is different from that of either adefovir dipivoxil or lamivudine, and that it may affect one or more essential steps or functions in HBV replication other than HBV DNA polymerase activity.

Example 2

Activity of Compound LY Against Lamivudine-Resistant and Lamivudine Sensitive Pre-Core (G1896A) Mutant Hepatitis B Viruses

The recombinant HBV-baculovirus/HepG2 assay system described below is employed in the following experiment to demonstrate that Compound LY exhibits antiviral activity against lamivudine-resistant and lamivudine-sensitive pre-core (G1896A) mutant hepatitis B viruses.

Concentrations of Anti-HBV Agents

Initially, Compound LY (Mitsubishi Pharma Corporation, Tokyo, Japan) or lamivudine (commercially available as 3'-thiacytidine) are dissolved in 100% dimethylsulfoxide (DMSO) to yield stock solutions with an initial concentration of 50 mM. These are stored at 4°C until just prior to dilution in media on the day of use. The compounds in DMSO are diluted in phosphate buffered saline (PBS, for example 137 mM sodium chloride, 10 mM phosphate, 2.7 mM potassium chloride, pH 7.4) to yield final concentrations in the range from 0.0001 μM to 10 μM.

Recombinant HBV Baculovirus Testing

Construction, generation, amplification, and purification of recombinant baculoviruses are performed as described in W.E. Delaney, IV, and H.C. Isom, Hepatitis B Virus Replication in Human HepG2 cells Mediated by Hepatitis B Virus Recombinant Baculovirus, *Hepatology* 28:1134-1146 (1998), using a wild-type 1.28 times genome length (genotype D). A panel of 10 recombinants is generated, with individual members encoding mutations which cause the following polymerase changes: none (wild-type [wt]); L526M; L526M + M550I ("dual I"); L526M + M550V ("dual V"); and M550I. The remaining five members are identical to those described above except that they also harbor the pre-core (G1896A) stop codon.

Baculovirus Transduction and Drug Treatments

HepG2 cells (American Type Culture Collection, Manassas, VA) are seeded into 60 mM dishes at a density of 2-3 x 10^6 cells per dish and are transduced with 100 plaque forming units (PFU) of HBV-baculovirus per cell approximately 24 hr post-seeding as described in W.E. Delaney, IV et al., Cross-Resistance Testing of Antihepadnaviral Compounds Using Novel Recombinant Baculoviruses Which Encode Drug-Resistant Strains of Hepatitis B Virus, *Antimicrob. Agents Chemother.*, 45(6):1705-13 (2001). Cells are incubated in medium containing the indicated concentrations of drugs with a medium change every 2-3 days for seven more days.

Analysis of HBV DNA

After seven days, media samples are collected and centrifuged at 2000 rpm for 5 minutes to pellet cellular debris. HBV particles are precipitated from 1.5 ml of media samples with 0.5 ml of 50% PEG 8000, mixed, and incubated overnight at 4°C as described in Delaney et al., *supra*. Particles are pelleted by centrifugation at 4°C for 20 minutes at 14000 g and resuspended in 100 µl of a buffer containing 10 mM Tris (pH 7.5), 5.5 mM $MgCl_2$. Samples are then digested with 20U of DNase 1 for 1 hour at 37°C and the reaction stopped by the addition of 200 µl of 2x X-DNA buffer (1x contains 100 mM Tris, pH 7.5, 1% SDS, 100 mM EDTA). The samples are then subjected to extraction and analysis by quantitative PCR as described below.

Intracellular HBV replicative intermediates are isolated from cytoplasmic core particles. *Id.* Cell monolayers are washed with PBS and then lysed with 750 µl of 0.5% Nonidet P-40 in PBS for 20 minutes at 4°C. Cell lysates are transferred to microcentrifuge tubes and centrifuged for 5 minutes to remove nuclei. The supernatants are transferred to fresh tubes, adjusted to 10 mM $MgCl_2$, and then incubated with 10U of DNase 1 for 1 hour at 37°C. The digestion mixture is then adjusted to 20 mM EDTA, 1% SDS, and 100 mM NaCl. At this stage, 200 µl of each sample are removed for extraction and analyzed by quantitative PCR. Proteinase K is then added to the remainder of each sample at a a final concentration of 0.5 mg/ml and

incubated at 37°C for 4 hours. Extraction with Tris-saturated phenol and then chloroform are performed, and DNA is recovered by incubation with an equal volume of isopropanol and then stored overnight at –20°C. DNA pellets are resuspended in 5 mM EDTA and digested with 20U of DNase-free RNase for 1 hour at 37°C before analysis by electrophoresis and Southern blotting. *Id.*

Quantitative PCR

Real time PCR using molecular beacons is employed to measure extracellular hepatitis B virion DNA quantitatively as described by S.R Lewin et al., Analysis of Hepatitis B Viral Load Decline Under Potent Therapy: Complex Decay Profiles Observed, *Hepatology* 34:1012-1020 (2001).

Quantitation of Antiviral Effects

Image densities obtained by Southern blotting are scanned and then quantified using Sigma Gel software as described by D. Colledge et al., In Vitro Antihepadnaviral Activities of Combinations of Penciclovir, Lamivudine, and Adefovir, *Antimicrob. Agents Chemother.*, 44(3):551-60 (2000). Data are analyzed using TableCurve2D (Jandel Scientific; San Rafael, CA) as described in Colledge et al., *supra*, to determine the particular effects on HBV replication in the assay.

The results of experiments in which the activity of Compound LY and, for comparison, lamivudine, are evaluated against lamivudine-resistant and lamivudine-sensitive HBeAg positive and negative (pre-core (G1896A) mutant HBV), as measured by Southern Blot of intracellular HBV DNA , are shown in Table 2.

TABLE 2. EFFECTS OF THE PRECORE G1896A MUTATION ON DRUG SENSITIVITY
Based on Southern Blot (Intracellular HBV DNA) Results

DRUG	Polymerase Change	IC_{50} (μM; mean ± S.E.M.) HBeAg$^+$ (wild-type)	IC_{50} (μM; mean ± S.E.M.) HBeAg$^-$ (PC[a] G1896A)	IC_{50} Ratio (PC:wt)	p Value[b]
COMPOUND LY					
	None	0.056 ± 0.007	0.033 ± 0.003	0.6	0.04
	L526M	0.303 ± 0.129	0.200 ± 0.064	0.7	NS
	L526M+M550I	0.693 ± 0.324	(0.03)#	(0.04)	-
	L526M+M550V	0.583 ± 0.429	0.247 ± 0.147	0.4	NS
	M550I	0.510 ± 0.51	0.143 ± 0.220	0.3	0.003
LAMIVUDINE					
	None	0.004 ± 0.003	0.005 ± 0.001	1.3	NS
	L526M	0.14 ± 0.038	0.053 ± 0.017	0.4	NS
	L526M+M550I	>10	>10	-	NS
	L526M+M550V	>10	>10	-	NS
	M550I	>10	>10	-	NS

[a]Pre Core

[b]Student "t" test; HBeAg positive compared to HBeAg negative HBV with same polymerase sequence

Only 1 replicate

As shown in Table 2, against wild-type (HBeAg positive), lamivudine-sensitive virus, Compound LY displays an IC_{50} of 0.056 ± 0.007 μM. Similarly, Compound LY is active against HBeAg negative, lamivudine-sensitive HBV, exhibiting an IC_{50} of 0.033 ± 0.003 μM. In these experiments, lamivudine is very potent against both of these viruses, exhibitng IC_{50}s against HBeAg positive and HBeAg negative, lamivudine-sensitive HBV of 0.004 ± 0.003 μM and 0.005 ± 0.001 μM, respectively.

Although Compound LY is less active against viruses containing mutations that confer resistance to lamivudine (L526M, L526M+M550I/V, and M550I), it exhibits approximately equal activity against viruses containing these same mutations whether they are HBeAg positive or negative. Compound LY displays an IC_{50} of 0.303 ± 0.129 μM against HBeAg positive HBV containing the L526M alteration, and has an IC_{50} of 0.200 ± 0.064 μM against the HBeAg negative HBV having this same alteration. Against viruses containing the L526M+M550I alterations, Compound LY displays an IC_{50} of 0.693 ± 0.324 μM and 0.03 μM against HBeAg positive and HBeAg negative HBV, respectively. Similarly, against viruses containing the L526M +M550V alterations, Compound LY displays IC_{50}s of 0.583 ± 0.429 and 0.247 ± 0.147 μM against HBeAg positive and HBeAg negative HBV, respectively. Against HBV containing the M550I alteration, Compound LY displays IC_{50}s of 0.510 ± 0.51μM and 0.143 ± 0.220 μM against HBeAg positive and negative HBV, respectively.

Lamivudine displays activity against HBeAg positive and negative viruses containing the L526M alteration in the HBV DNA polymerase with an IC_{50} of 0.14 ± 0.0038 and 0.053 ± 0.017 μM against HBeAg positive and negative HBV, respectively. As expected, lamivudine is not active against HBeAg positive or negative viruses containing the other alterations in the viral DNA polymerase (L526M+M550I/V, and M550I), displaying an IC_{50} of greater than 10.

The results of experiments in which the activity of Compound LY and, for comparison, lamivudine, are evaluated against lamivudine-resistant and lamivudine sensitive HBeAg positive and negative (pre-core (G1896A) mutant), as measured by QPCR of extracellular HBV virion DNA , are shown in Table 3.

TABLE 3. EFFECTS OF THE PRECORE G1896A MUTATION ON DRUG SENSITIVITY
Based on QPCR (Extracellular Virion DNA) Results

DRUG / Polymerase Change	IC_{50} (μM; mean ± S.E.M.) HBeAg$^+$ (wild-type)	IC_{50} (μM; mean ± S.E.M.) HBeAg$^-$ (PC[a] G1896A)	IC_{50} Ratio (PC:wt)	p Value[b]
COMPOUND LY				
None	0.008 ± 0.006	0.006 ± 0.002	0.75	NS
L526M	0.077 ± 0.038	0.077 ± 0.015	1	NS
L526M+M550I	0.457 ± 0.258	(0.010#)	(0.02)	-
L526M+M550V	0.740 ± 0.560	0.046 ± 0.027	0.06	NS
M550I	(5.40)#	0.393 ± 0.142	0.07	-
LAMIVUDINE				
None	0.001 ± 0.000	0.0007 ± 0.0004	0.7	NS
L526M	0.160 ± 0.150	0.025 ± 0.010	0.16	NS
L526M+M550I	>10	>10	-	NS
L526M+M550V	>10	>10	-	NS
M550I	>10	>10	-	NS

[a]Pre Core

[b]Student "t" test; HBeAg positive compared to HBeAg negative HBV with same polymerase sequence

Only 1 replicate

As shown in Table 3, against wild-type (HBeAg positive), lamivudine-sensitive virus, Compound LY displays an IC_{50} of 0.008 ± 0.006 μM. Similarly, Compound LY is active against lamivudine-sensitive, HBeAg negative HBV with an IC_{50} of 0.006 ± 0.002 μM. Again, in these experiments, lamivudine is very potent against both of these viruses, with IC_{50}s against HBeAg positive and HBeAg negative HBV of 0.001 ± 0.000 μM and 0.0007 ± 0.0004 μM, respectively. Compound LY is active against

viruses containing mutations that confer resistance to lamivudine (L526M, L526M+M550I/V, and M550I), whether they are HBeAg positive or negative. Compound LY displays an IC_{50} of 0.077 ± 0.038 μM against HBeAg positive HBV containing the L526M alteration and is equally active (IC_{50} of 0.077 ± 0.015 μM) against HBeAg negative HBV having the same alteration. Against viruses containing the L526M+M550I alterations, Compound LY displays an IC_{50} of 0.457 ± 0.258 μM and 0.010 μM against HBeAg positive and HBeAg negative HBV, respectively. Against viruses containing the L526M +M550V alterations, Compound LY displays IC_{50}s of 0.740 ± 0.560 μM and 0.046 ± 0.027 μM against HBeAg positive and HBeAg negative HBV, respectively. Against HBV containing the M550I alteration, Compound LY displays IC_{50}s of 5.4 μM and 0.393 ± 0.142 μM against HBeAg positive and negative HBV, respectively.

Lamivudine displays activity against HBeAg positive and negative HBV viruses containing the L526M alteration in the HBV DNA polymerase (IC_{50} = 0.160 ± 0.150 μM and 0.025 ±0.010 μM against HBeAg positive and negative HBV, respectively). However, lamivudine is not active against HBeAg positive or negative HBV viruses containing the other alterations in the viral DNA polymerase (L526M+ M550I/V, and M550I). Against any of these viruses, lamivudine displays an IC_{50} of greater than 10.

Conclusions

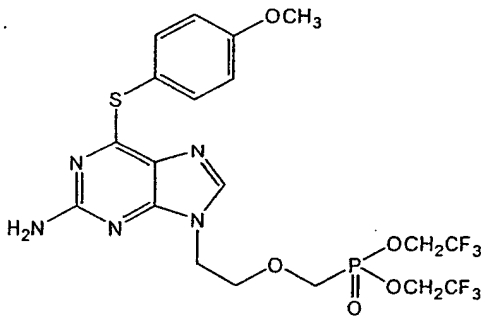
Taken together, the results shown in Tables 2 and 3 and described above demonstrate that in the absence of other mutations, the pre-core (G1896A) stop codon mutation, which confers an HbeAg negative phenotype, has no significant effect on HBV susceptibility to Compound LY or lamivudine. Pre-core mutants that have alterations in the viral DNA polymerase that confer resistance to lamivudine have a decreased sensitivity to Compound LY up to 12-fold. However, this decrease is small in concentration terms, and not statistically significant (based on assays for intracellular HBV DNA).

In general, when compared to wild-type HBV, hepatitis B viruses that contain the pre-core mutation (G1896A) appear to be slighly more susceptible to Compound LY and lamivudine. This observation is not fully understood, especially considering that the G1896A alteration appears to increase viral replication efficiency.

The invention being thus described, it is obvious that the same can be varied in many ways. Such variations are not to be regarded as a departure from the spirit and scope of the present invention, and all such modifications as would be obvious to one skilled in the art are intended to be included within the scope of the following claims.

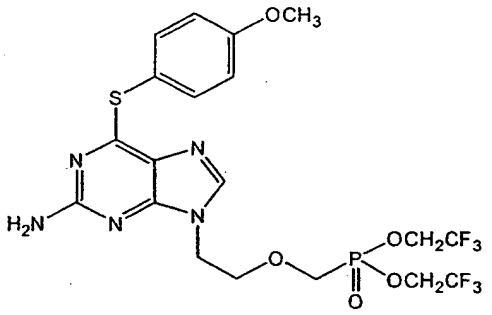
We Claim:

1. A method for preventing or treating pre-core mutant hepatitis B virus disease, comprising administering to a patient in need thereof an antiviral effective amount of a phosphonate nucleotide compound having the structure:



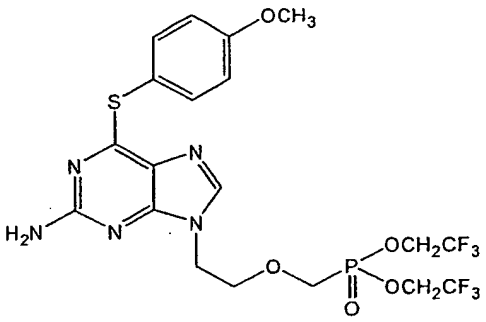
or a pharmaceutically acceptable salt, hydrate, or solvate thereof.

2. A method for preventing or treating lamivudine-resistant, pre-core mutant hepatitis B virus disease, comprising administering to a patient in need thereof an antiviral effective amount of a phosphonate nucleotide compound having the structure:



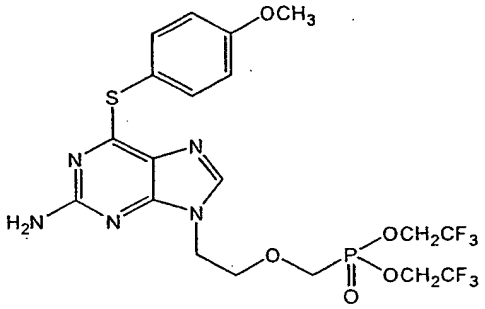
or a pharmaceutically acceptable salt, hydrate, or solvate thereof.

3. Use of a compound having the structure:



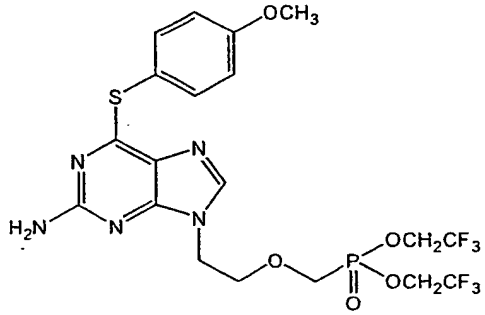
or a pharmaceutically acceptable salt, hydrate, or a solvate thereof, to prevent or treat pre-core mutant hepatitis B virus disease.

4. Use of a compound having the structure:



or a pharmaceutically acceptable salt, hydrate, or solvate thereof, to prevent or treat lamivudine-resistant, pre-core mutant hepatitis B virus disease.

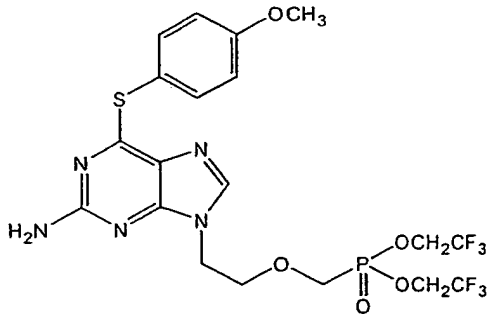
5. Use of a compound having the structure:



to prepare a medicament for the prevention or treatment of pre-core mutant hepatitis B

virus disease in a human patient.

6. Use of a compound having the structure:



to prepare a medicament for the prevention or treatment of lamivudine-resistant, pre-core mutant hepatitis B virus disease in a human patient.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 03/06711

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K31/7064 A61P31/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07F A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, MEDLINE, EMBASE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	ONO-NITA SUZANE KIOKO ET AL: "Novel nucleoside analogue MCC-478 (LY582563) is effective against wild-type or lamivudine-resistant hepatitis B virus." ANTIMICROBIAL AGENTS AND CHEMOTHERAPY. UNITED STATES AUG 2002, vol. 46, no. 8, August 2002 (2002-08), pages 2602-2605, XP002245846 ISSN: 0066-4804 the whole document abstract --- -/--	1-6

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 June 2003	11/07/2003

Name and mailing address of the ISA	Authorized officer
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Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
P,X	COLLEDGE, COLACINO: "The purine nucleotide analogue LY582563 (mcc-478) inhibits replication of wild type and drug resistant hepatitis b virus" JOURNAL OF HEPATOLOGY, vol. 36, no. suppl.1,307A, April 2002 (2002-04), page 88 XP002245854 abstract	1-6